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INFLUENCE OF STEROIDS ON FIBROBLASTS. II. THE FIBROBLAST AS AN ASSAY SYSTEM FOR TOPICAL ANTI-INFLAMMATORY POTENCY OF CORTICOSTEROIDS*

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Increasing experimental evidence points to cortisol in contradistinction to cortisone, as a potent anti-inflammatory agent (1–3). Studies involving closely related compounds differing only in the oxygen function at the 11-position have indicated that this position is of prime importance in the suppression of fibroblast growth (4–6), and that in this situation also, steroids with the 11β-OH configuration are the active ones. Moreover, the homologous 11-desoxy compounds not only are fibroblast inhibitors of intermediate strength *in vitro* (4), but also *in vivo* (7). Since the cellular composition of connective tissue is over 90% fibroblasts, it is not too surprising that inflammatory responses and fibroblast responses parallel one another. That the action of fibroblasts in an isolated tissue culture system can also be correlated to inflammatory responses (8) is a fortunate occurrence for studying basic tissue-steroid interactions.

The sensitivity of the tissue culture fibroblast to naturally occurring and synthetic steroids is a consistent, measurable phenomenon (4, 5, 9–11); in fact, it is considered a most sensitive target cell (12, 13) for steroids. in other studies (14), it has been shown that different tissue culture cell types respond in a variety of ways to the same steroid. Certain positions on the corticosteroid molecule, of which the 11-position is but one, have long been known to be of critical importance for topical anti-inflammatory activity (2, 7, 15, 16). It is now also known that protecting or potentiating these key structures by addition of various substitutions to the steroid molecule enhances its topical antiphlogistic activity (2, 15, 16).

The data presented here are correlations between growth inhibition by steroids in a tissue culture fibroblast test and topical anti-inflammatory activity.

MATERIALS AND METHODS

Murine fibroblasts (929-L) were grown as monolayers in Eagle's minimum essential medium (17) (Earle's balanced salt solution), containing 2 mM glutamine, 10% calf serum, penicillin (100 U/ml) and streptomycin (0.1 mg/ml). Roller tubes 18 × 150 mm, containing 5 ml of media, were inoculated on day zero of the experimental period with 100,000 cells. Following a 24 hour stationary phase to allow cell attachment to glass surfaces, the tubes were rotated four days at ⅕ revolution per minute. On day five, cells were scraped free of the glass by rubber policemen, mixed 10 times in an automatic syringe, and diluted to give cell suspensions of convenient concentration for counting. Details of the method are described in a previous paper (20).

The initial cell inoculation was made into media containing either 1 μL/ml of propylene glycol (control) or steroid dissolved in 1 μL/ml of propylene glycol. Purified steroid was weighed and diluted with absolute methanol to a concentration of 100 μg/ml. Appropriate aliquots of this solution or 100- or 1000-fold dilutions of it were added to a propylene glycol-methanol mixture in 8 ounce prescription bottles. The methanol was evaporated under a nitrogen stream so that a final, water soluble steriod solution was assured in the propylene glycol culture medium.

Determinations of number of cells per roller tube were performed in an electronic cell counter‡ adjusted to count all particles larger than 975 μ. Five tubes were utilized to determine each point, and the resulting data subjected to statistical analysis by means of analysis of variance and regression calculations (18, 19). Logarithmically transformed data were graphed as average number of cells per tube on the ordinate, against steroid dose as the abscissa. Relative potencies were calculated for the compounds tested, based on an assigned potency of one for cortisol. Criteria for statistical accuracy were thoroughly checked (20).

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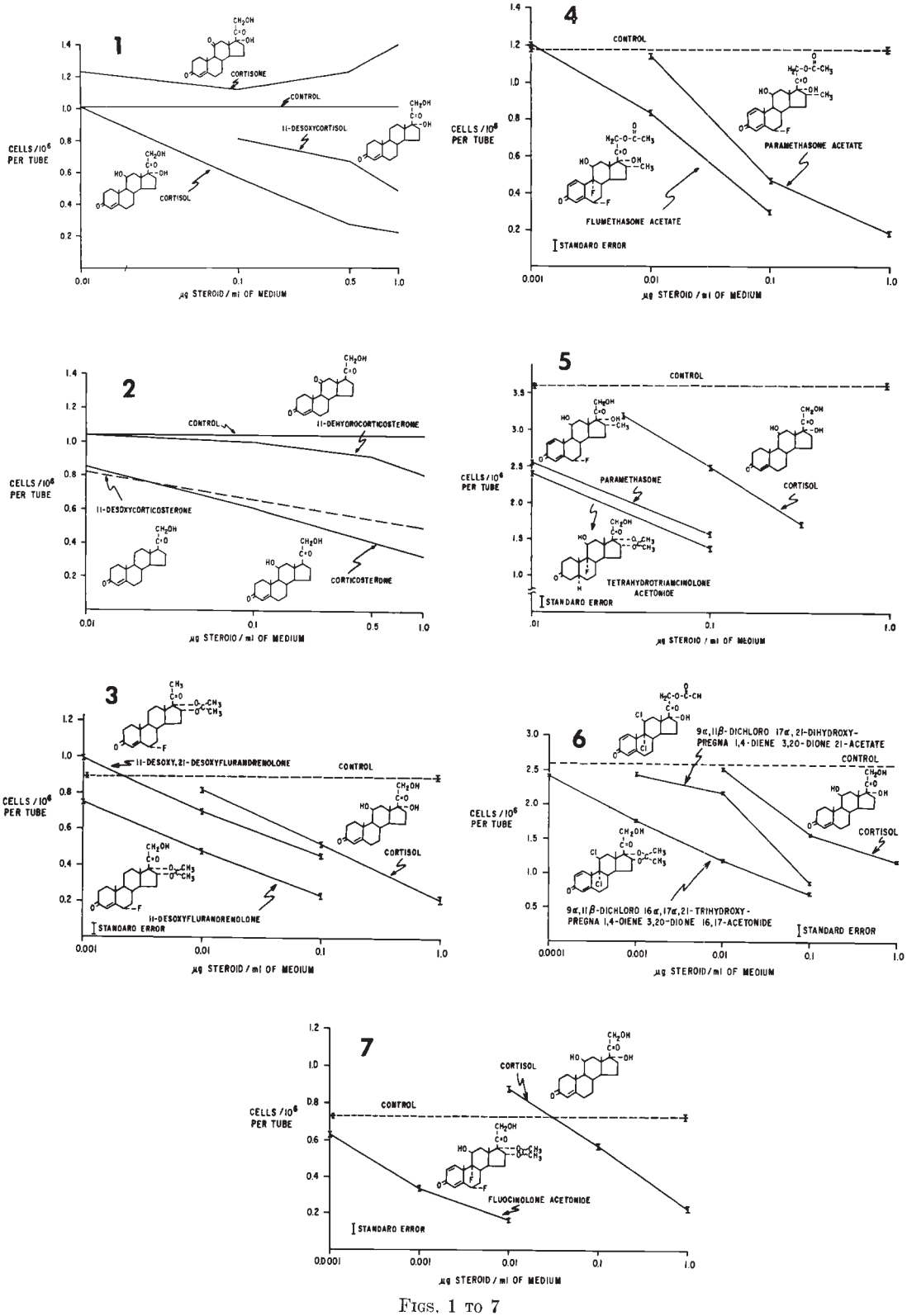
‡ Coulter Model B.


1
CORTISONE
CONTROL
11-DESOXYCORTISOL
CORTISOL
CELLS / 10⁶ PER TUBE
μg STEROID / ml OF MEDIUM
2
CONTROL
11-DEHYDROCORTICOSTERONE
11-DESOXYCORTICOSTERONE
CORTICOSTERONE
3
11-DESOXY, 21-DESOXYFLURANDRENOLONE
CONTROL
CORTISOL
11-DESOXYFLURANDRENOLONE
STANDARD ERROR
4
CONTROL
PARAMETHASONE ACETATE
FLUMETHASONE ACETATE
STANDARD ERROR
5
CONTROL
PARAMETHASONE
CORTISOL
TETRAHYDROTRIAMCINOLONE ACETONIDE
STANDARD ERROR
6
9α,11β-DICHLORO 17α, 21-DIHYDROXY-PREGNA 1,4-DIENE 3,20-DIONE 21-ACETATE
CONTROL
CORTISOL
9α,11β-DICHLORO 16α,17α,21-TRIHYDROXY-PREGNA 1,4-DIENE 3,20-DIONE 16,17-ACETONIDE
STANDARD ERROR
7
CORTISOL
CONTROL
FLUOCINOLONE ACETONIDE
STANDARD ERROR

FIGS. 1 TO 7

The index of significance (g) varied between 0.004 and 0.01, indicating significant slope regression for all fibroblast inhibiting steroids. Indices of precision (λ) varied between 0.11 and 0.39, but the vast majority were below 0.2 (20). Therefore, the variability within the experimental method was extremely small. Moreover, all experiments were repeated at least once, with good reproducibility of potency ratios obtained.

RESULTS

The steroid vehicle, propylene glycol, was tested for effects on cell growth at a number of concentrations. At 1 μL/ml, the concentration used in the experiments, there was no inhibition of cell growth. These experiments are reported in detail in other publications (5, 20).

Figure 1 is a composite of two experiments illustrating the effect on fibroblast growth of the closely related compounds cortisone (11-keto), cortisol (11β-OH), and 11-desoxycortisol. The logarithmic abscissa represents dose of steroid, the ordinate millions of cells per tube with each point based on an average of 5 tubes. The lines connect actual points (for easier visualization), and are not the fitted lines obtained by calculation. Cortisol is an inhibitor of fibroblast growth at 0.1, 0.5, and 1.0 μg/ml (approximately 3×10^{-7} to 3×10^{-6} M) of medium. Cortisone, on the other hand, does not inhibit fibroblasts *in vitro*, and has a stimulatory effect at all concentrations tested. However, only at 1.0 μg/ml is this statistically significant. The compound of the set which has no oxygen at position C-11 is intermediate in action between the other two compounds.

Figure 2 is a similar representation in an experiment involving 11-dehydrocorticosterone (ketone at C-11), corticosterone (β-OH at C-11), and 11-desoxycorticosterone. Again, the compound containing a ketone at position C-11 does not inhibit the growth of tissue culture fibroblasts except at the highest concentration, whereas the 11β-OH compound is the most potent of the set, and the compound without an 11-oxygen function is intermediate in action. Similar results were found in experiments using 2-methylcortisone and 2-methylcortisol, as well as with prednisone and prednisolone (4).

Figure 3 illustrates the dose-response curves obtained when tissue culture fibroblasts are incubated with the synthetic compounds 11-desoxyflurandrenolone (6α-fluoro 16α,17α,21-trihydroxy-pregn-4-ene-3,20-dione-16,17-acetonide) and 11-desoxy, 21-desoxyflurandrenolone (6α-fluoro 16α,17α-dihydroxy pregn-4-ene-3,20 dione, 16,17-acetonide). By referring to cortisol as a standard, it is apparent that both compounds are active fibroblast inhibitors, but 11-desoxyflurandrenolone is the more potent.

Figure 4 is a graphic representation of the actions of paramethasone acetate (6α-fluoro 16α-methyl-11β,17α,21-trihydroxy, pregna-1,4-diene 3,20-dione 21-acetate) and flumethasone acetate (6α,9α-difluoro, 16α-methyl-11β,17α, 21-trihydroxy pregna-1,4-diene 3,20-dione 21-

FIG. 1. Two cycle semilogarithmic plot showing action of cortisone, 11-desoxycortisol, and cortisol on fibroblast growth. Millions of cells, based on an average from five tubes per point, are plotted on the ordinate against steroid concentration on the abscissa.

FIG. 2. Two cycle semilogarithmic plot showing action of 11-dehydrocorticosterone, 11-desoxycorticosterone, and corticosterone on fibroblast growth. Millions of cells, based on an average from five tubes per point, are plotted on the ordinate against steroid concentration on the abscissa.

FIG. 3. Three cycle semilogarithmic plot showing action of cortisol, 11-desoxy, 21-desoxyflurandrenolone, and 11-desoxyflurandrenolone on fibroblast growth. Millions of cells, based on an average from five tubes per point, are plotted on the ordinate against steroid concentration on the abscissa.

FIG. 4. Three cycle semilogarithmic plot showing action of flumethasone acetate and paramethasone acetate on fibroblast growth. Millions of cells, based on an average from five tubes per point, are plotted on the ordinate against steroid concentration on the abscissa.

FIG. 5. Two cycle semilogarithmic plot showing action of cortisol, paramethasone and tetrahydrotriamcinolone acetonide on fibroblast growth. Millions of cells, based on an average from five tubes per point, are plotted on the ordinate against steroid concentration on the abscissa.

FIG. 6. Four cycle semilogarithmic plot showing action of dichlorisone, dichlorisone 16,17-acetonide, and cortisol on fibroblast growth. Millions of cells, based on an average from five tubes per point, are plotted on the ordinate against steroid concentration on the abscissa.

FIG. 7. Four cycle semilogarithmic plot showing action of cortisol and fluocinolone acetonide on fibroblast growth. Millions of cells, based on an average from five tubes per point, are plotted on the ordinate against steroid concentration on the abscissa.

acetate) on the fibroblast. Flumethasome acetate markedly depresses growth at 0.01 (3×10^{-8} M) and 0.1 μg/ml (3×10^{-7} M), while the other compound is active at 0.1 and 1.0 μg/ml. It has been our experience that a steroid and its acetate behave in a similar fashion in the tissue culture fibroblast assay because of the presence of an acylase in this system. In Figure 5 the relationship between paramethasone and tetrahydrotriamcinolone acetonide (9α-fluoro 11β,16α,17α,21-tetrahydroxy allopregnan 3,20-dione 16,17-acetonide) is seen as that of a weaker to a more potent compound, but both are far more potent than cortisol.

In Figure 6, the potencies of two different 9,11-dichloro steroids are depicted. Dichlorisone acetate (9α,11β-dichloro-17α,21-dihydroxypregna 1,4-diene,3,20-dione 21-acetate) and dichlorisone 16,17-acetonide are more active than cortisol, but the 16,17-acetonide steroid inhibits fibroblast growth at a concentration of 0.001 μg/ml (3×10^{-9} M).

In Figure 7, the potency of fluocinolone acetonide (6α,9α-difluoro 11β,16α,17α,21-tetrahydroxy pregna 1,4-diene 3,20-dione 16,17-acetonide) is well illustrated. This compound significantly inhibits fibroblast growth at a concentration of 0.001 μg/ml (3×10^{-9} M) and is several hundred times as powerful as cortisol in this activity. In separate experiments, fluocinolone acetonide has markedly inhibited fibroblast growth at a concentration of 0.00075 μg/ml and moderately inhibited it at 0.0005 μg/ml (approximately 10^{-9} M). The structural formulae of all the steroids tested and their potency ratios are presented in Table I.

CONCLUSIONS

The compounds presented in Table I are depicted in order of increasing strength as fibroblast inhibitors. Corticosterone has a relative potency of 0.48 when compared to cortisol. The diminished fibroblast inhibition demonstrated by corticosterone is due to its lack of an α-OH group at the C-17 position, such as is possessed by cortisol. The importance of the 11-position, however, is overriding, as can be seen in the case of cortisone, which exhibits no fibroblast inhibition, despite the 17α-hydroxyl group. A ketone at position C-11 completely blocks the growth depressing effect of the steroid and, in fact, can confer a growth enhancing potential (4) on the molecule, as is the case with cortisone.

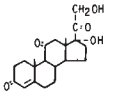
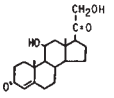
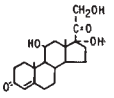
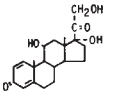
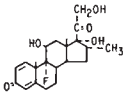
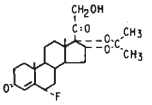
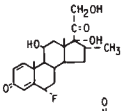
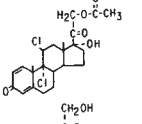
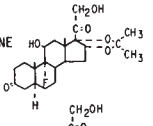
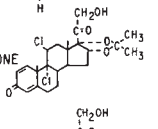
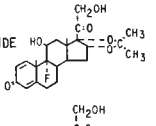
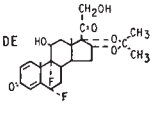
It is known that this steroid, when parenterally administered, has anti-inflammatory activity only when it can be transformed to cortisol (3). In the tissue culture system under discussion, the biotransformation of cortisol and cortisone (11β-OH dehydrogenase system) is minimal (4), and that is the reason for the dichotomy between the *in vivo* and *in vitro* activities of cortisone. In dermatological practice also, topical cortisone is disappointingly devoid of anti-inflammatory activity (2, 25).

The fourth compound listed in Table I, prednisolone, differs from cortisol only in the presence of an additional double bond in ring A between C-1 and C-2. Its fibroblast inhibiting potency is essentially the same as that of cortisol. The table shows the relative potencies of these compounds measured by the fibroblast assay method, as well as their relative potencies with other assays. The oral potency of prednisolone is about 4 times that of cortisol (26). In contrast, however, topical activity is only one or two times that of cortisol (2, 27). Here again, as in the case of cortisone, the agreement between steroidal topical anti-inflammatory activity and the fibroblast assay, is very close.

Dexamethasone is 7.5 times more potent in suppressing fibroblast growth than cortisol or prednisolone. This characteristic is attributed to the addition of a 9α-fluoro and a 16α-methyl substitution to the molecule. Dexamethasone has been found highly effective orally as an anti-inflammatory agent (26). It is interesting to note that of all of the assays listed, only the tissue culture fibroblast system finds a potency for dexamethasone which is comparable to topical clinical trial (2, 28, 29). Paramethasone differs from dexamethasone in the position of the α-fluorine group. Paramethasone, with the α-fluorine at position 6, is somewhat more potent than dexamethasone.

Reference to the lower part of the table shows that the 16,17-acetonide group is a potent enhancer of fibroblast inhibitory activity to the steroid molecule. Yet 11-desoxyflurandrenolone, which possesses this substituent, is less potent than paramethasone.

TABLE I

STEROID	STRUCTURAL FORMULA	POTENCY RATIOS				
		FIBROBLAST ASSAY (Average of > 2 Determinations)	TOPICAL ANTIPHLOGISTIC TEST (16)	ANTIGRANULOMA ACTIVITY (21,22)	THYMUS INVOLUTION ASSAY(*16,21,22)	TOPICAL CLINICAL EVALUATION (2,23,24)
CORTISONE			0.58		*0.62	
CORTICOSTERONE		0.48	0.33		*0.17	
CORTISOL		1.0	1.0	1.0	1.0	1
PREDNISOLONE		1.7	3.4	2.7	4.0 *4.1	2
DEXAMETHASONE		7.5	38	104.0	47.0 *83.	10
11-DESOXYFLURAN-DRENOLONE		7.8				
PARAMETHASONE		11.3		63.6	45.1	
Δ',9,11-DICHLOROCORTISONE ACETATE		14.2				
TETRAHYDROTRIAMCINOLONE ACETONIDE		19.4				
Δ',9,11-DICHLOROCORTISONE 16,17-ACETONIDE		43.7	23.2		<1	
TRIAMCINOLONE ACETONIDE		156.		48.5	37.7	40
FLUOCINOLONE ACETONIDE		440.		446.0	263.0	160

The reason for this can be found in the 11-position. As was seen in Figures 1 and 2, the absence of the 11β-OH causes a marked loss of fibroblast growth depression.

Dichlorisone, which is similar to cortisol except for an additional double bond in ring A and chlorine substitutions at positions C-9 and C-11, has a potency in the same range in the fibroblast assay as parametha sone and dexamethasone. In the thymolytic and anti-

granulomatous assays, its activity is the same or less than that of cortisol (30). Tolksdorf refers to data which indicates that dichlorisone has topical anti-inflammatory activity higher than prednisolone in humans (30).

Tetrahydrotriamcinolone acetonide, a steroid in which both double bonds at C-1,2 and C-4,5 are reduced, leaving a 3-ketone, 5-α configuration in ring A, surprisingly enough possesses fibroblast inhibiting capacities. This is the first instance in which we have found a steroid which is reduced in ring A, with any activity (5). Compared to 11-desoxyflurandrenolone, it is approximately twice as potent, further indicating that the 11β-OH group is crucial to the fibroblast inhibiting activity of the steroid molecule. The presence of a 16,17-acetonide structure in these fluorinated steroids can override the diminished potency secondary to reduction in ring A.

Dichlorisone 16,17-acetonide, which is also a 9α,11β-chlorinated steroid, has a potency ratio of 43.7 by the fibroblast assay (Table I). This compound has been shown to be less active than cortisol in the thymolytic test (16), but 23.2 times as potent as cortisol by a topical antiphlogistic test (16). The parenteral-topical discrepancy for these two 9,11-dichloro steroids is dramatic. However, their potency by the fibroblast assay is very similar to their topical anti-inflammatory activity.

Triamcinolone acetonide possesses only one fluorine group, but it also has one of the most potent functional groups, the 16,17-acetonide, which confers upon it a potency of 156 times that of cortisol.

The strongest fibroblast growth suppressant tested by us to date, and also the most potent topical anti-inflammatory agent (2, 23, 24) is fluocinolone acetonide. This compound causes consistent, profound depression of fibroblasts at extremely low concentrations (5) (see Fig. 6) and has a relative potency of 440 when compared to cortisol.

The presence of a 16,17-acetonide confers on the steroid molecule a high degree of fibroblast inhibition, and topical anti-inflammatory potency. This is well illustrated by reference to the potency ratios of three sets of compounds differing only in the absence of a 16,17-acetonide structure. Thus, dexamethasone has a relative potency of 7.5, while triamcinolone acetonide has a potency of 156. Likewise, dichlorisone and dichlorisone 16,17-acetonide have potency ratios of 14.2 and 43.7, respectively. Finally, fluocinolone acetonide is 22 times more potent as a fibroblast inhibitor than flumethasone (P.R. = 20).

All the naturally occurring steroids tested by the fibroblast inhibition assay method were used in concentrations well within physiological limits. By extrapolating from the relative potency data, it is possible to recognize that the synthetic steroids are in the same concentration range. Hence, one of the criticized shortcomings of *in vitro* testing 31) is eliminated; namely, that concentrations used are of such toxic proportions that they can give no information concerning *in vivo* action. The thymus involution and antigranulomatous assays have always been useful for general evaluation of corticosteroid activity. However, they do not always correlate well with topical potency. Moreover, as can be seen by reference to Table I, the findings of the fibroblast assay method are in excellent agreement with clinical findings of topical anti-inflammatory activity. The method, therefore, can be an extremely useful addition to the bioassays available for preclinical trial purposes.

The need for a test that correlates well with topical anti-inflammatory activity is further illustrated in the case of another steroid, 16α, 17α - dihydroxyprogesterone - 16,17 - acetonide, which has been shown by Bronstein et al. 32) to be without efficacy in treating human skin inflammations, despite strong potency by several animal antigranuloma assays. In the fibroblast assay also, this compound is without significant inhibitory activity within physiological concentrations. However, the addition of a fluorine at position-6 confers fibroblast inhibitory activity on this compound, as can be seen in Figure 3.

It is in the elucidation of structure-activity relationships that the fibroblast assay is of greatest utility. It is apparent from the data presented in this paper that an interdependent hierarchy of functional groups exists. Steroids require 17α- and 21-hydroxyl groups for enhanced topical anti-inflammatory activity. However, the 11-position plays a role that is vital. A β-OH group at the C-11 position

gives fibroblast inhibition of marked potency, and the mere removal of two hydrogens at this position destroys the inherent inhibitory action of the entire molecule. In the synthetics, the situation is more complex, but it can be said that added 6α- and 9α-fluorines, and formation of 16,17-acetonides are the methods of conferring increasing fibroblast growth inhibitory action on the steroid nucleus.

SUMMARY

A number of naturally occurring and synthetic steroids were tested for fibroblast inhibiting potency in a tissue culture system. It was found that the Δ^4, 3-ketone, 21-hydroxyl, and 11β-hydroxyl groups are necessary to the steroid nucleus for insuring fibroblast inhibition. The 17α-OH group is also of importance in this regard. Increasing steroidal fibroblast depression is attained by addition of complex substituents such as 6α- and 9α-fluorines, and presence of a 16,17-acetonide structure on the steroid molecule.

Fibroblast inhibiting potency and topical anti-inflammatory activity are possessed by 9α,11β-dichloro compounds, despite their lack of potency in anti-granuloma and thymus involution tests. Comparison of steroid potency measured by this test and by other laboratory procedures to topical anti-inflammatory activity manifest by clinical trial, indicated that the tissue culture assay is an excellent addition to the bioassay systems available.

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